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Article

Immune sexual dimorphism during acute infection by *Toxocara canis*: A novel approach for an old infection

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Abstract: *Toxocara canis* is a helminth parasite with importance in public and veterinary health. Although different studies have evaluated the immune response, to date there are no studies where it is analyzed from the point of view of sexual dimorphism. At 7 days of infection, female rats had a higher number of larvae in the liver, while male rats had them in the lungs. The percentages of immune cells were evaluated, and in most cases, no significant differences were observed. Regarding the cytokines production, infection can generate a decrease in Th1 such as IL-1β in both sexes and IL-6 only in females. In the case of Th2, IL-4 increases only in infected males and IL-5 increases in males and decreases in females due to the effect of infection. IL-10 also decreases in both sexes as a consequence of the infection and TGF-β only in females. Finally, the infection generates the production of antibody against the parasite, however, their quantity is lower in females. Thus, this study demonstrates that *Toxocara canis* infection is dimorphic and affects females more than males. This is due to a polarization of the inadequate immune response, which is reflected as a higher parasite load in this sex.

Keywords: sexual dimorphism; *Toxocara canis*; toxocariasis; immune response; public health; zoonoses.

1. Introduction

Immune sexual dimorphism can be defined as the differences in the immune response that exist when the organism is exposed to an antigenic challenge caused by a virus, bacteria or parasite. These differences have been associated with the role of hormones such as sex steroids that play an important role in the regulation of the immune system [1–5]. During different studies where the immune response against helminths has been evaluated. Previous studies have evaluated the influence of hormones on the immune function, and, it has been observed that the sex (male or female) plays an important role in the regulation of the immune response, hence it has been generally demonstrated that one of two sexes with which one sex become is more susceptible than another to suffer different parasite infections [6–9]. In some parasitic infections, a hormonal regulation has been associated with larval reactivation that are vertically transmitted (transplacental, lactogenic) from mother to offspring, as is the case of Toxocariasis [10,11]. However, so far it has not been described whether there is a dimorphic effect on the immune response that could influence the migration of the larvae towards organs such as the uterus and the mammary gland, hence modifying susceptibility of males and females.

Toxocara species are parasitic nematodes whose definitive hosts are dogs and cats. *Toxocara canis* (*T. canis*) infects dogs as definitive host, whereas *Toxocara cati* (*T. cati*) is

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found in cats. In addition, this parasite can affect humans; therefore, it is considered a zoonotic disease; as well as other mammals, birds and invertebrates among others, causing the disease known as Toxocariasis [10,12,13]. In humans, the infection can be asymptomatic or can produce different clinical manifestations caused for the migration of *T. canis* larvae through the bloodstream and other organs. This disease can be classified as Visceral Larva Migrans (VLM), Neurotoxocariasis (NT), Ocular Larva Migrans (OLM), and Covert Toxocariasis (CT) [14–16].

During *T. canis* infection, the predominant immune response is a Th2 type-one, although a dichotomy has also been mentioned in terms of the polarization of the immune response; as it is possible to find molecules of both the Th1 and/or Th2 type. This response may be influenced by aspects such as; host species (dog, cat, mouse, rat, human), time of infection (acute or chronic), and target organ (lung, liver, central nervous system), among others. However, it is worth mentioning that, currently, the immune response during *T. canis* infection has not been analyzed from asexual dimorphism perspective. In general, the immune response is assembled against somatic antigens and excretory-secretory antigens (TcES-Ag) [17–19]. This response is characterized by a strong adaptive immune response, where a Th2-type response predominates during the chronic infection. The Th2 response is characterized by the production of cytokines such as interleukin (IL) IL-4, IL-5, IL-6, IL-13, IL-33, and regulatory cytokines such as IL-10 among others [17–21]. However, during the acute phase of infection, a strong pro-inflammatory response takes part of the immunological profile, where a mixed response with inflammatory cytokines of innate origin, together with Th17 and Th2 type cytokines has been reported [22].

Th2 cytokines are involved in the activation of other immune cell subpopulations such as mast cells, macrophages, eosinophils, and the secretion of Immunoglobulins (Ig) IgE and IgG1 [18,20]. These cytokines and Ig are associated with leukocytosis, peripheral blood eosinophilia, eosinophilic infiltration around larval sites of migration, as well as specific antibody production [22].

Macrophages also play an important role during infection, and macrophages obtained from mice within ten days of infection with *T. canis*, and cultured *in vitro* with lipopolysaccharide, have been reported to produce higher amounts of IL-10 and tumor growth factor-beta (TGF-β), and lower amounts of Th1 cytokines such as IL-12 and tumor necrosis factor-alpha (TNF-α) [23]. Later, macrophages that display a different phenotype from the classical ones, namely, alternatively activated macrophages (AAMs), are involved in the initiation of immune responses against helminths, as well as in tissue repair [24]. In addition, TcES-Ag can modulate the immune response by inducing the formation of T and B regulatory cells, which also produce IL-10 and TGF-β [25], the former being one of the strategies that help parasites evade the host immune response.

In addition, different studies have shown that the immune system can be regulated by different hormones, such as sex steroids [26–30]. On this basis, differences in susceptibility and the associated immune response have also been reported in some other parasitic diseases [6–8,31–35]. Nonetheless, as far as we are concerned, there is a lack of information that demonstrates the immune modulation caused by sex steroids during Toxocariasis. Within this framework, this study aims to evaluate susceptibility, immune response, and immune effector mechanisms during the acute *T. canis* infection from the point of view of immune sexual dimorphism, to describe an immunomodulatory effect that that could increase susceptibility to infection to this parasite in male or females.

2. Materials and Methods

2.1 Experimental animals

Fourteen male and fourteen female Wistar rats of sixty days old, were obtained from the experimental animal facility of the Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México. Rats were divided into four groups and allocated in polycarbonate boxes (50 cm L x 23 cm W x 21 cm H). The groups were set as follows: I)

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Control males (♂ Ctrl *n*=7), II) Infected males (♂ Infx *n*=7), III) Control females (♀ Ctrl *n*=7), and IV) Infected females (♀ Infx *n*=7). Animals were kept in cycles of 12 hours light/darkness. Water and food were provided *ad libitum* in sterile conditions.

2.2 Ethic statement

The protocol for the use and care of the animals was endorsed by both Institute´s Animal Care and Use Committee, (Comité de Cuidado y Uso de Animales de Experimentación, CICUAL, permit number 201-2016) following the official Mexican regulations (NOM-062-ZOO-1999), which are in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institute of Health of the USA, to ensure compliance with established international regulations and guidelines.

2.3 Collection and processing of T. canis eggs

T. canis eggs were obtained from adult parasites donated by Centro de Control Canino de Cuautitlán, Estado de México. Worm females were separated, rinsed in tap water, and placed in phosphate buffer (PBS) with a pH around 7.2 - 7.4. Afterward, uteri were collected through an incision in the first third of the body and placed in a physiological saline solution. Eggs were obtained using a fine pore filter. The ova were washed several times in PBS and centrifuged at 3250g / 5 min. Finally, the eggs from the pellet were resuspended in a PBS / 2% formaldehyde solution and incubated at 27°C for 28 days to obtain 80-90% of the infective form (larvated eggs) of the parasite.

2.4 Rats infection

Male and female rats were infected with 1,000 *T. canis* larvated eggs at sixty days old. The inoculum for the infection was washed three times to eliminate the PBS/formaldehyde solution. Eggs were then resuspended in PBS and concentrated to 1,000 larvated eggs per ml. Infection was performed by intragastric administration using a type Foley metallic probe. Seven days later, the rats were humanely euthanized using sevoflurane (Sevorane®) anesthetic overdose.

2.5 Serum obtention

Right after the rats were euthanized, blood was collected in SST-vacutainer serum tubes. After collection, blood was allowed to clot by leaving it undisturbed at room temperature for 3 minutes and clot was removed by centrifuging at 2,000 x g for 10 min in a refrigerated centrifuge. Following centrifugation, serum was immediately transferred into a clean glass tube using a Pasteur pipette. Serum samples were maintained and stored at –20°C until further analysis.

2.6 Larvae recovery from lung and liver

Lungs and liver of infected rats were weighed and macerated; tissue was digested in artificial gastric juice [1% pepsin (250 units/mg, SIGMA®) and 1% HCl 37%. pH: 2.0 (10 ml of artificial gastric juice / 1 gr of tissue)] for 24 hours. Samples were centrifuged at 791 g / 5 min, and the pellet was resuspended in 1 ml 4% paraformaldehyde. Parasite counting involved 10 counts per 20 µl sample, the total number of larvae was multiplied x 50 to calculate the number of larvae per ml and therefore the number of larvae per gram of tissue.

2.7 Flow cytometry assay

For the assay, spleen, mesenteric lymph nodes (MLN), inguinal, and axillar peripheral lymph nodes (PLN) were collected from rats immediately after euthanasia. The samples were disaggregated using a sterile nylon mesh (50 µm) and a syringe plunge in PBS at 4°C. The splenic cell suspension was centrifuged at 182 g/3 min, decanted, and resuspended in 500 µl of ACK buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH

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7.3) for 10 min at room temperature to lyse erythrocytes present in the sample; afterwards, 700 µl of FACS buffer was added followed by centrifugation at 182 g/3 min. All samples (Spleen and lymph nodes cells) were washed with PBS (phosphate saline solution), fixed with 4% paraformaldehyde for 10 min/37°C; and furtherly resuspended in 500 µl FACS buffer. Finally, cells were transferred to 96-well plates (Costar®).

For extracellular staining, cells were incubated with a primary antibody solution for 10 min / 4°C. The following selected primary antibodies were used: AF 488 anti-rat CD3 (Biolegend. Clone 1F4) (1:100), PE/Cy5 Mouse anti-rat CD4 (BD bioscience. Clone Ox-35) (1:300), PE Mouse anti-rat CD8α (BD bioscience. Clone Ox-8) (1:200), PE anti-rat CD45RA (Biolegend. Clone Ox-33) (1:200), PE anti-rat TCRγδ (Biolegend. Clone V65) (1:200), AF647 anti-rat CD161 (Biolegend. Clone 10/78) (1:200) and anti-rat CD11b/c-biotin (Biolegend Clone Ox-42) (1:200) + PE/Cy5 Streptavidin (Biolegend) (1:200). Primary antibodies were set in 3 different groups: T1; AF 488 anti-rat CD3 + PE/Cy5 Mouse anti-rat CD4 + PE Mouse anti-rat CD8α. T2; AF 488 anti-rat CD3 + PE anti-rat CD45RA + anti-rat CD11b/c-biotin. T3; AF647 anti-rat CD161 + PE anti-rat TCRγδ. Cells were washed twice with 150 µl FACS buffer after incubation, resuspended in 200 µl of FACS buffer, and stored at 4° C in the dark. Cell analysis was performed with an Attune NEXT (Applied Bioscience®) flow cytometer in the National Laboratory of Flow Cytometry. Data analysis was performed using FlowJo software v10.0 (Treestar Inc.®). Isotype controls were used for each of the organs analyzed to prepare the reading gates in the cytometer.

2.8 Cytokine production determination

Eight different cytokines: IL-1β, IFN-γ, TNF-α, IL-4, IL-5, IL-6, IL-10 and IL-13, were measured using the MILLIPLEX®MAP Rat Cytokine/Chemokine Magnetic Bead Panel (RECYTMAG-65K) (Merck KGaA, Darmstadt, Germany). TGF-β1 was determined with MILLIPLEX®MAP TGF-β1 Single Plex Magnetic Bead Kit (TGFBMAG-64K-01) (Merck KGaA, Darmstadt, Germany). Frozen serum samples were thawed and prepared according to manufacturer instructions. Median fluorescence intensity was obtained from duplicates of serum samples using a MAGPIX from LUMINEX by Millipore facilitated at the Unidad de Investigación from Facultad de Medicina Veterinaria y Zootecnia, UNAM. Obtained data was further analyzed with Myassays software package using the Four Parameter Logistic Curve fitting method to calculate the sample cytokine concentration.

2.9 T. canis antigens purification and quantification

TcES-Ag was obtained by culturing the larvae according to the method described by Savigny (De Savigny, 1975) and modified by Bowman (Bowman et al., 1987). The integrity and purity of antigens were determined by SDS-PAGE and Coomassie staining. Protein content was quantified by the Bradford method (Bradford, 1976). Different TcES-Ag dilutions were performed in PBS and mixed with the Bradford reagent. The protein concentration was determined by comparing the optical density (O.D.) of the sample in a standard curve (10, 20, 40, 60, 80, and 100µg / ml) made with bovine serum albumin (SIGMA®). The concentration of total antigens obtained in the test was 235.14 µg / ml.

2.10 Specific anti-T. canis IgG determination

To determinate specific antibodies, 96 wells flat-bottom polystyrene plates (Maxisorp, NUNC Labs) were sensitized with 1 µg/ml TcES-Ag in bicarbonate buffer (pH 9.6) for 24 hours at 4° C. Subsequently, plates were washed three times with 0.01% PBS-Tween 20, blocked with 3% bovine serum albumin (BSA), and stored at 4° C for 24 hours. After blocking time finished, the plates were washed three times with PBS-Tween 20 at 0.01% and stored at 4° C. For each sample, 50 µl of serum/well was used in duplicate, diluted 1:200 with PBS and incubated at 37° C for two hours. Subsequently, plates were washed three times with PBS-Tween 20 at 0.01%, and 50 µl/well of secondary antibody [1:1,000 peroxidase-conjugated AffiniPure Goat Anti-Rat IgG (H+L)] was added and incubated at

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37° C for one hour. Plates were washed five times with the same solution (PBS-Tween 20 at 0.01%) and color development was achieved by using 0.05% o-phenylenediamine (OPD) and 0.001% hydrogen peroxide in citrate buffer. The plates were incubated for 15 min in total darkness at 37°C, and then, 50 µl/well of 0.06% orthophosphoric acid was added. Plates were read at 492 nm with 15 seconds of agitation in an ELISA reader (Stat Facs 4200).

2.11 Statical analysis

Data are presented as mean ± standard deviation (SD). Results are shown in bar graphs that describe mean and standard deviation. Analyses were performed with Prism V 8.0 software (GraphPad Software Inc®). The experimental design considers 2 independent variables: sex [male (♂) or female (♀)] and infection [Control (Ctrl) or *T. canis* infection (Infx)]. Data regarding parasite charges (larval recovery) only consider the infection variable. These values were evaluated by a Two-way ANOVA and a *post-hoc* Bonferroni multiple comparison test between all groups. A significant difference was considered when $p<0.05$.

3. Results

3.1. Macroscopic lesions and larvae number during acute T. canis infection

The number of lesions and larvae number was significantly different depending on sex. In males, lesions and larvae counts were higher in the lungs seven days post-infection. In contrast, in females, the extension of the lesions and the number of recovered larvae were higher in the liver (Figure 1A, B**)**. Combining the mean number of larvae obtained in both organs we were able to observe statistically significant differences ($P<0.001$). In males, we recovered a total of 94.6 larvae (28.7 in the liver + 65.9 in the lung), while in females, 119 larvae were collected (95.5 in liver + 23.5 in lungs) (Figure 1B).

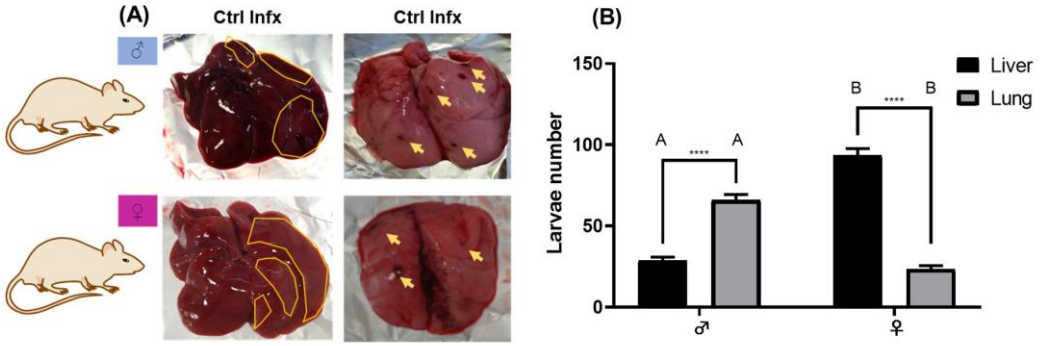

Figure 1. Macroscopic lesions and larvae number of *T. canis* in the lungs and liver. (**A**) Liver and lungs injuries. In liver, the areas of damage are outlined in yellow. In lungs, yellow arrows point to areas of damage. (**B**) Total larvae number in both organs and larvae number in liver and lung. Bars represent the mean ± SD of larvae number. Letters (A, B) show the significant differences between sexes.

3.2. Immune system cells subpopulations

3.2.1. Innate immune cells

Because sex steroids are able to modulate the immune response in infections, we decided to evaluate the immune response during a *T. canis* infection as an antigenic challenge. Figure 2 shows Macrophages (Mϕ), Natural Killer cells (NK) and T gamma-delta

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lymphocytes (TγδL) in spleen, PLN and MLN. In spleen, differences were only seen in Mϕ percentage between males and females, as the percentage of these cells was higher in the former, regardless of the infection status ($p<0.05$) (Figure 2A**)**. In the PLN, there were no statistically significant differences in the subpopulations analyzed that could be attributed to sex or to an infection condition ($p>0.05$). In male-collected MLN, infection generated an increase in the percentage of Mϕ (Figure 2G).

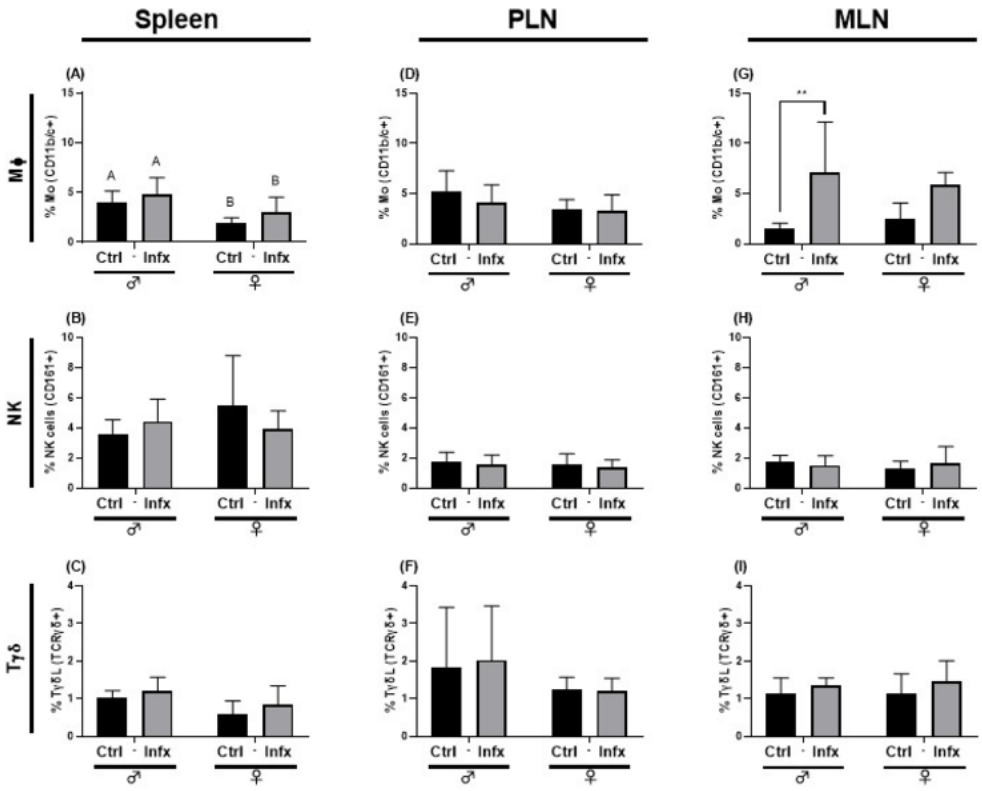

Figure 2. Innate immune cells subpopulations. Determination of innate immune subpopulations by flow cytometry. (**A**) Mϕ (CD11b/c$^+$) in spleen. (**B**) NK cells (CD161$^+$) in spleen. (**C**) Tγδ L (TCRγδ$^+$) in spleen. (**D**) Mϕ (CD11b/c$^+$) in PLN. (**E**) NK cells (CD161$^+$) in PLN. (**F**) Tγδ L (TCRγδ$^+$) in PLN. (**G**) Mϕ (CD11b/c$^+$) in MLN. (**H**) NK cells (CD161$^+$) in MLN. (**I**) Tγδ L (TCRγδ$^+$) in MLN. Bars represent the mean ± SD of percentage of immune cells. Asterisks show the significant differences between Ctrl and Infx animals and the letters (A, B) between sexes.

3.2.2. Adaptative immune cells

Figure 3 shows the percentage of total T cells, T helper lymphocytes (ThL) and T cytotoxic lymphocytes (TcL) in spleen, PLN and MLN. In spleen, the infection generated an increase in the percentage of ThL only in males ($p<0.05$) (Figure 3B). In the PLN, the percentage of ThL was higher in control females in contrast to control males ($p<0.05$), yet when the infection was present in females, this percentage decreased ($p<0.0001$) (Figure 3E). Finally, total T cell percentages in MLN were higher in females than in males. A statistically significant difference was observed in control ($p<0.001$) and infected ($p<0.01$) animals. (Figure 3G). Regarding ThL, the infection causes a decrease in the percentage of these cells in both sexes ($p<0.05$) (Figure 3H).

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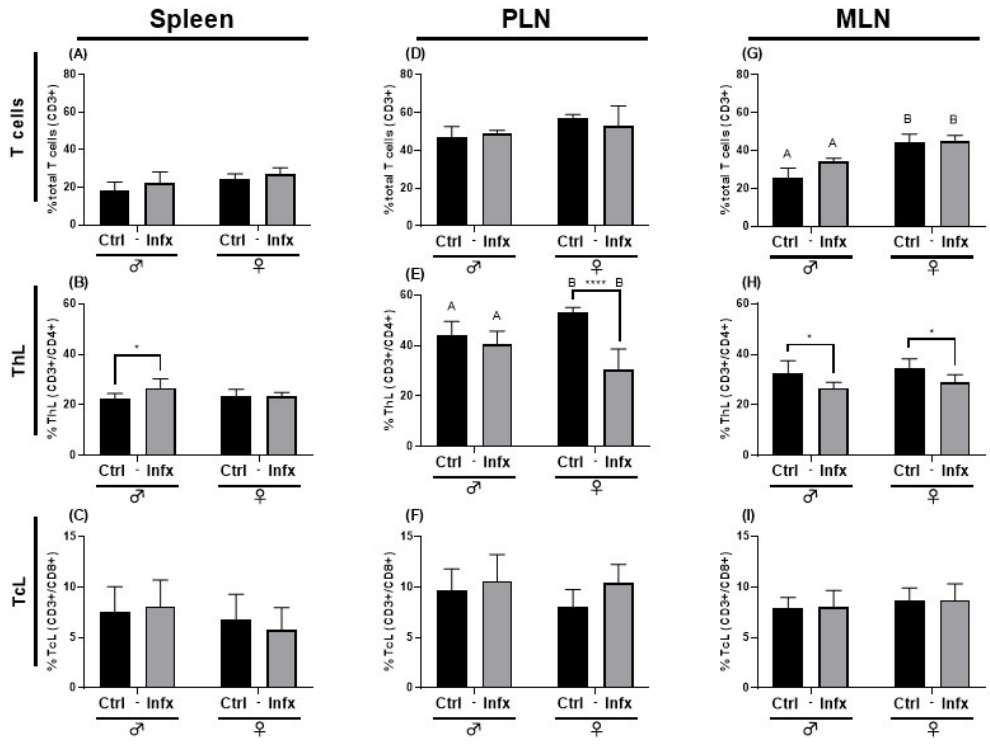

Figure 3. T lymphocytes. Determination of T cells subpopulations by flow cytometry. (**A**) total T cells ($CD3^+$) in spleen. (**B**) ThL ($CD3^+/CD4^+$) in spleen. (**C**) TcL ($CD3^+/CD8^+$) in spleen. (**D**) total T cells ($CD3^+$) in PLN. (**E**) ThL ($CD3^+/CD4^+$) in PLN. (**F**) TcL ($CD3^+/CD8^+$) in PLN. (**G**) total T cells ($CD3^+$) in MLN. (**H**) ThL ($CD3^+/CD4^+$) in MLN. (**I**) TcL ($CD3^+/CD8^+$) in MLN. Bars represent the mean ± SD of percentage of immune cells. Asterisks show the significant differences between Ctrl and Infx animals and the letters (A, B) between sexes.

Finally, in B lymphocytes (BL), the infection generates an increase in the number of these cells ($p<0.01$) in females PLN (Figure 4B).

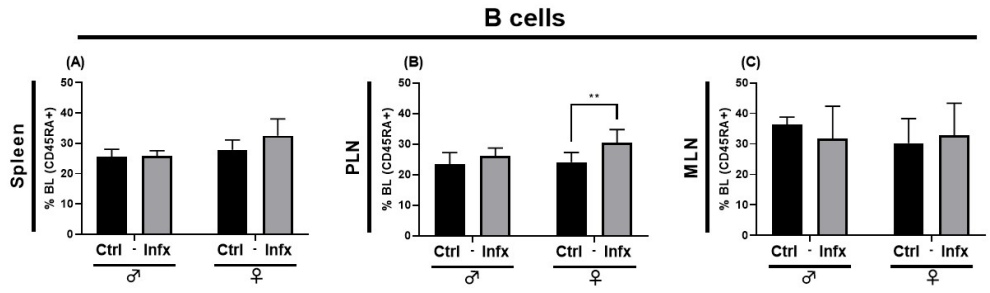

Figure 4. B Lymphocytes. Determination of B cells subpopulations by flow cytometry. A, BL ($CD45RA^+$) in spleen. B, BL (CD45RA+) in PLN. C, BL (CD45RA+) in MLN. Bars represent the mean ± SD of percentage of immune cells. Asterisks show the significant differences between Ctrl and Infx animals.

3.3. Cytokine production

To evaluate cell response in the immune system, we analyzed the production of cytokines during *T. canis* infection in both sexes. For Th1 cytokines, the infection reduces the concentration of IL-1β in both sexes ($p<0.0001$). In addition, the levels of this cytokine are

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lower in females compared to males in both the control ($p<0.05$) and the infected group ($p<0.01$) (Figure 5A). IFN-γ concentration also shows a dimorphic pattern where males have higher levels than females ($p<0.01$) (Figure 5B). TNF-α was not detected in any of the experimental groups (Figure 5C). IL-6 levels are higher in control females ($p<0.05$), but when during an infection, the cytokine levels decrease ($p<0.001$) (Figure 5D). Regarding Th2 cytokines, infection causes an increase of IL-4 ($p<0.01$), IL-5 ($p<0.01$), and IL-13 ($p<0.05$) levels but only in males (Figure 5E-G). Sex-related differences in IL-5 levels were observed, as generation of this cytokine was higher in males than in females during infection ($p<0.01$) (Figure 5E). In contrast, IL-5 levels are higher in control females than in males; interestingly, infection did not increase theses IL-5 levels ($p<0.01$) (Figure 5F). IL-13 shows no significant differences due to sex ($p>0.05$) (Figure 5G). Likewise, regarding regulatory cytokines, there are no differences related to sex ($p>0.05$), but the infection causes a decrease in the expression of IL-10 in both sexes ($p<0.0001$) (Figure 5H) and TGF-β decreases only in females ($p<0.05$) (Figure 5I).

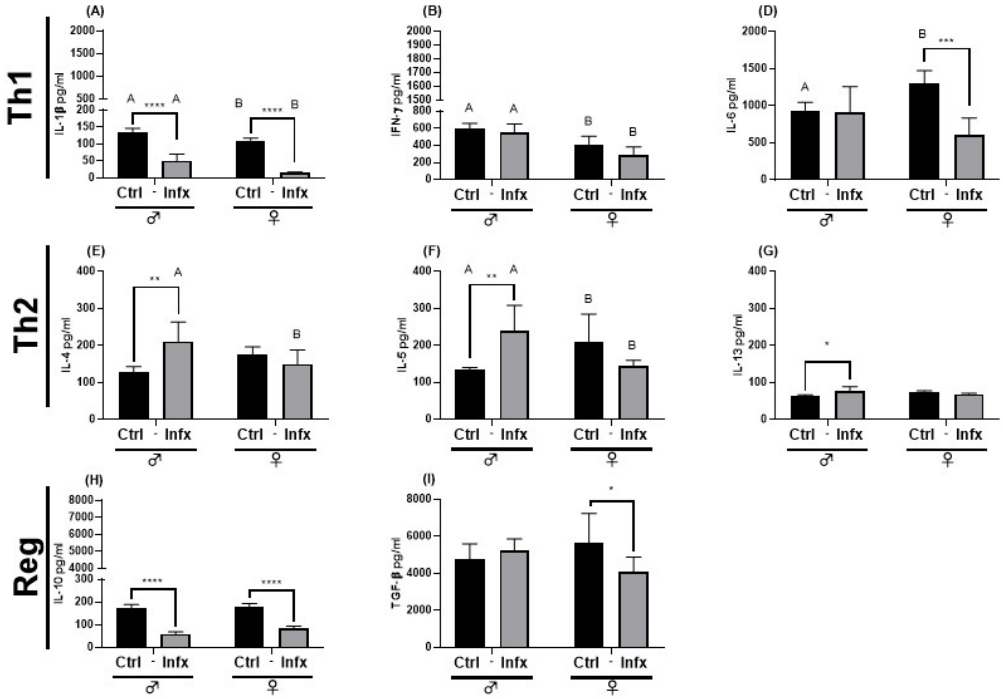

Figure 5. Analysis of systemic soluble factor expression in serum. Cytokine measurement was performed in serum using the MILLIPLEX Magnetic Bead Panel. (**A**) IL-1β. (**B**) IFN-γ. (**C**) TNF-α. (**D**) IL-6. (**E**) IL-4. (**F**) IL-5. (**G**) IL-13. (**H**) IL-10 and (**I**) TGF-β. Bars represent the mean ± SD of cytokines concentration. Asterisks show the significant differences between Ctrl and Infx animals and the letters (A, B) between sexes.

3.4 Specific anti-T. canis IgG production

Since the production of specific antibodies is an important strategy to the control of helminths, we decided to determine the presence of these antibodies in the serum of the animals. The infection caused an increase in the production of specific Ig-G anti-*T canis* in both sexes ($p<0.0001$), but in females, this increase is lower than in males ($p<0.01$) (Figure 6).

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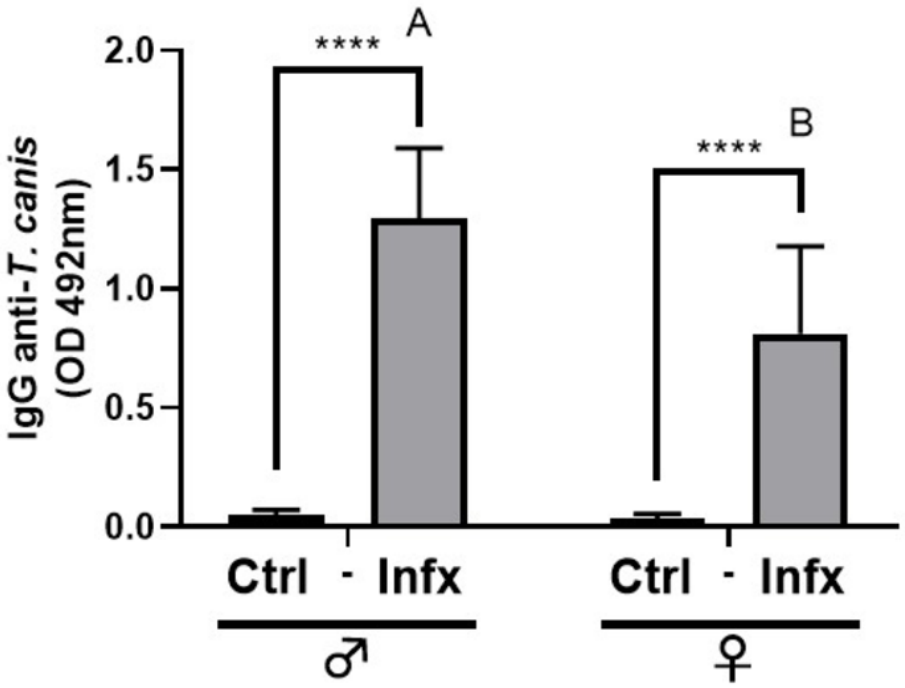

Figure 6. Specific anti-*T. canis* antibody production in control and infected male and females. The titration was performed by ELISA. Bars represent the mean ± SD of IgG antibodies levels. Asterisks show the significant differences between Ctrl and Infx animals and the letters (A, B) between sexes.

4. Discussion

Authors For a long time, infectious diseases were considered to affect males and females equally. However, different mechanisms have now been described and current evidence has shown that infectious processes, such as diseases caused by parasites, have a dimorphic component in which one of the sexes is more affected than the other. These differences may be due to the effects that hormones such as sex steroids have on the regulation of the immune response [4,7,26,28,36], or even to a possible direct effect of hormones on physiologic processes of parasites [5,9,37–40].

In this work, the effect of sex on susceptibility to *T. canis* infection was estimated by evaluating the immune response during the acute phase of the infection in a rat model. The first point to assess susceptibility to infection focused on the analysis of parasitic loads in organs such as lungs and liver. The evaluation was carried out in these organs since, at the time of infection, migrating somatic larvae are present throughout these organs [12,41]. We found the first dimorphic difference at seven days post-infection, a mean of 28.7 larvae (2.87% of total larvated eggs) were collected from livers of males and 65.9 from lungs of males (6.59% of total larvated eggs); whereas in females, we found a mean of 93.5 larvae in the liver (9.53% of total larvated eggs) and 23.5 (2.35% of total larvated eggs) in lungs (Figure 1B). Our data are similar to those reported by Lescano *et. al.,* (2004), who infected male Wistar rats with 500 larvated eggs, and observed that the number of larvae recovered at eight days post-infection in the liver and lung was 12.3 (2.46% of total larvated eggs) and 35.7 (7.14% of total larvated eggs), respectively [41]. Studies with other species of *Toxocara* (*T. cati*), by Santos *et. al.,* (2009) using adult male Wistar rats infected with 300 larval eggs, reported a recovery of 4 larvae (1.33% of total larvated eggs) from the liver and 13.6 larvae (4.53% of total larval eggs) from the lung [42]. Further experiments from dos Santos *et. al.,* (2017) evaluated the pattern of somatic larvae migration in male and female Wistar rats infected with 300 larvated eggs. They report that seven days post-infection, the recovery of larvae in males was 13 larvae in the liver (4.33% of total larvated eggs) and 2 in the lungs (0.66% of total larvated eggs). In females, the number of

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larvae recuperated from liver is 27.5 (9.16% of total larvated eggs) and 1.5 in lungs (0.5% of total larvated eggs). The increase in the number of larvae at the hepatic level in females was maintained from seven to 60 days post-infection. In addition, the total number of larvae recovered in these organs was higher in females than in males, demonstrating that females are more susceptible than males to *T. canis* infection. [43]. These results are similar to those reported by our research group, where we observed that the number of larvae in the liver and lung has a dimorphic distribution, as females have a higher number of larvae in the liver than males; although these differences in the parasitic loads at the pulmonary level shows statistically significant differences, where males have a greater number of larvae than females. In addition, the total number of larvae in both organs was higher in females than in males (Figure 1B).

Since the immune response plays a key role in the development of resistance or susceptibility to different diseases of non-infectious or infectious etiology, we decided to evaluate the primary cells of the innate and adaptive immune response responsible for the control of helminth infections in spleen, MLN, and PLN. Regarding cells from the innate response, only Mϕ showed differences in their percentage at the splenic level, where females presented a lower percentage of these cells than males, regardless the presence of an infection or not (Figure 2A). In MLN, the infection causes an increase in the percentage of these cells in males, but not in females (Figure 2G). Mϕ have been proved to be important cells in the regulation of the immune response against helminths, being competent antigen-presenting cells, thus these changes in percentages could influence the susceptibility to develop an infection [20,32,44]. Although in our experiments, we did not elucidate the polarization of Mϕ towards an AAM phenotype, different studies have shown that females have a higher number of Mϕ than males [4,28]. Nonetheless, we did not confirm this finding in the current study. Regarding adaptive immune cells of females, infection causes a reduction in the number of ThL in PLN and MLN (Figure 3E, H), whereas in males, these cells increase in spleen but decrease in MLN (Figure 3B, H). Likewise, Nava-Castro *et. al.,* (2019) demonstrated that male and female BALB/c mice during acute infection (5 days post-infection) with *Trichinella spiralis*, present a decrease in the percentage of ThL in MLN [45], yet a different antigenic challenge was used in the referred trial. Similar experiments from Ruiz-Manzano *et. al.,* (2020), who used a model of chronic *T. canis* infection in females, showed no differences in the percentage of splenic ThL in infected animals compared to the control group. However, no differences in PLN were demonstrated opposed to our results [18]. BL are crucial cells in the control of nematodes, since they mature into plasma cells and produce specific antibodies against these parasites. In BL, we observed an increase in the percentage due to the infection in PLN of females (Figure 4B). In MLN, there were no significant changes in both sexes; which also agrees with reports published by Nava-Castro (2019), where no differences were seen in the percentage of these cells during the *Trichinella spiralis* acute phase of the infection [45]. In contrast, during chronic *T. canis* infection, the percentage of these cells increases in the spleen and PLN of females [18]. When differences in cell subpopulations are evaluated, it must be considered that there is a lack of information in the literature on the impact of sex differences on the immune response during Toxocariasis. In addition, other factors must be considered, such as the time of infection, type of host, and the number of ingested larval eggs , among others, which makes the analysis even more complicated.

As mentioned above, the Th2 response plays an important role for the orchestration of the immune response during helminthic infections. Here, we report a decrease of Th1 cytokines; IL-1β, in both sexes and IL-6 only in females due to infection (Figure 5 A, D). Th2 cytokines IL-4, IL-5, and IL-13 are increased only in males due to this same effect (Figure 5 E-G). The regulatory cytokine IL-10 shows lower levels due to infection in both sexes (Figure 5H), while TGF-β only decreases in females (Figure 5I). Contrary to what we reported, Pecinali *et. al.,* (2005) found in male BALB/c mice infected with approximately 1,000 larval eggs of *T. canis*, increased levels of IL-6 and IFN-γ cytokines in plasma and bronchoalveolar fluid [46]. Th2 cytokines follow a pattern of increased production equal

to that reported when there is an infection by helminths only in males, the sex in which almost all studies evaluating the immune response to this parasite have been carried out [18,20,22,47]. Regarding IL-10 production, Torina *et. al.,* (2005) showed in pregnant bitches, that IL-10 levels increase while IFN-γ levels decrease during the first week of pregnancy [48]. These differences in IL-10 levels may be due to the fact that in the aforementioned trial, dogs were naturally infected, hence the exact phase of infection, namely the acute or chronic period of toxocariasis when IL-10 levels were determined, was uncertain. In contrast, the current study was done in a paratenic host during the acute phase of the infection. Finally, the production of specific anti-*T. canis* antibodies is lower in females as opposed to males (Figure 6). Different studies have shown that *T. canis* infection generates the production of specific IgG antibodies against the parasite [18,49,50], but so far, the production of antibodies anti-*T. canis* between males and females has not been analyzed.

These differences highlight the importance of studying infections from the point of view of immune sexual dimorphism. Although some research groups have been working for a long time on the study of parasitic diseases from a dimorphic point of view, this element is lacking in many other parasitic diseases. This groundbreaking research topic provides a new approach to the study of Toxocariasis, showing that host sex influences the development of the disease. In the light of all this, it is reasonable to state that sexual dimorphism influences parasite establishment in different hosts as well as its mode of transmission. It is important to highlight that this parasite can also be transmitted during pregnancy and/or lactation.

It is worth mentioning that further studies are needed to analyze other crucial cells for the development of immunity against helminths, such as innate lymphoid cells type 2 (ILCs2), the polarization of Mϕ towards an alternatively activated phenotype, the differentiation of T lymphocytes towards a Th2 profile and BL towards plasma cells. Furthermore, we cannot ignore the possible effect of hormones on parasite physiology, to better understand differences in disease susceptibility between males and females.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1.

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